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Original Article

Evaluation of the protective effect of *Zataria multiflora* Boiss essential oil on biochemical and histopathological parameters of kidney tissues in chronic administration of CCl_4

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ABSTRACT

Introduction: Carbon tetrachloride (CCl_4) is a toxic compound since it causes acute and chronic toxicity in various tissues due to oxidative stress. On the other hand, *Zataria multiflora* Boiss (ZM) essential oil as a natural product has different biological effects such as antioxidant activity. Therefore, this study was carried out to assess the protective potential of ZM essential oil on possible toxicity induced by chronic administration of CCl_4 in kidney tissues of rats.

Methods: Male Sprague-Dawley rats were assigned into five groups including C: control group; CO: vehicle control group; CE: rats that were given the essential oil (500µl/kg/day); F: rats that received CCl_4 (1ml/kg) twice a week; and FE: rats that were given CCl_4 and essential oil with the mentioned doses. After 11 weeks of study, kidney tissues were collected to measure the activity of AST, ALT, ALP, GGT and LDH enzymes and oxidative stress parameters (TAC, TBARS and GSH).

Results: The results showed a significant increase in the activity of ALT, ALP and LDH enzymes in kidney tissues of group F compared to the control groups, probably due to defects in cell metabolism induced by CCl_4. But in FE group, essential oil due to antioxidant activity could ameliorate the mentioned parameters in comparison to group F. There was not a significant change in the level of lipid peroxidation marker in kidney tissues of group F in comparison to the control groups. Histopathological studies also did not show any significant changes among kidney tissues of groups.

Conclusion: Administration of CCl4 affected on the activity of some biochemical enzymes in kidney tissues but there was no oxidative stress or injury in the tissues. However, prophylactic administration of ZM Boiss essential oil had antioxidant properties in modulating the measured parameters.

Keywords:
CCl_4
Kidney
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Introduction

The kidneys as vital organs of the body, are exposed to various injuries. These organs are very sensitive to toxic materials because they filter and reabsorb large amounts of blood (Safhi, 2018). One of the important factors in the pathophysiology of kidney injury is oxidative stress and its related mediators (Ozbek, 2012). A rise in the level of oxidants in the body creates a condition that is referred to as oxidative stress. The main sources of oxidants are endogenous, such as the production of free radicals as a byproduct of metabolic processes, and exogenous, such as environmental toxins (Phaniendra et al., 2015). One of these environmental toxins is carbon tetrachloride (CCl_4). It is produced in large quantities for industrial uses as a solvent for oils and fats, as a refrigerant, and as a dry-cleaning agent. Therefore, individuals may be exposed to CCl_4 by inhalation, ingestion or skin absorption (Faroon, 2005). Its chronic administration in laboratory animal models induces hepatic, renal and pulmonary fibrosis and damage to cardiac and testicular tissues.

Some researchers have applied the intra-gastric, intraperitoneal and subcutaneous administration of CCl_4 to induce chronic liver damage in animal models in order to assess the hepatic fibrosis pathogenesis (Teixeira-Clerc et al., 2006; Constandinou et al., 2005; Abraham and Wilfred, 1999). The tissue damage caused by CCl_4 depends on the administered dose and the duration of the exposure (Jayakumar et al., 2008; Pääkkö et al., 1996). The damage is caused by CCl_4 conversion to CCl_3• and CCl_3O_2• radicals by hepatic cytochrome P450 enzymes (CYP450). These free radicals can induce lipid peroxidation in membranes, resulting in cell and tissue damage in many organs, including the liver and kidneys (Weber et al., 2003; Rincón et al., 1999). In addition to increasing lipid peroxidation products, CCl_4 can decrease the antioxidant enzymes in the kidney (Doğukan et al., 2003). Some researchers have observed many histopathological and biochemical changes in hepatotoxicity and nephrotoxicity induced by CCl_4, such as the elevated activity of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) enzymes in serum due to leakage out of the damaged liver cells as well as the increased activities of renal and testicular lysosomal enzymes (Srivastava and Shivanandappa, 2010).

On the other hand, there are several reports that reveal traditional medicinal plants have potential antioxidants which are effective for prevention of oxidative stress-induced chronic diseases (Hassan et al., 2017). *Zataria multiflora* (ZM) Boiss is one of these traditional plants from the Lamiaceae family, which is native to Iran, Pakistan and Afghanistan (Zargari et al., 1990). Recent pharmacological studies have shown that ZM has a wide range of biological properties, including antimicrobial (Dini et al., 2015), anti-inflammatory (Boskabady and Gholami Mhtaj, 2014) and anticoagulant properties (Can Baser, 2008). Some researchers have discussed two phenolic compounds such as thymol and carvacrol as main ingredients of this essential oil with antioxidant activity (Shomali et al., 2016; Alizadeh and Shaabani, 2014; Sharififar et al., 2011; Sharififar et al., 2007). According to what was mentioned above, the present study was conducted to evaluate the protective effect of ZM Boiss essential oil on possible CCl_4 damages on kidney tissues by measuring oxidative stress parameters such as total antioxidant capacity (TAC), thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) as well as the activity of AST, ALT, LDH, ALP and gamma-glutamyl transferase (GGT) enzymes.

Material and methods

Materials

CCl_4 was purchased from Sigma (St. Louis, Mo, USA). Commercial kits for AST, ALT, GGT, ALP and LDH were purchased from Pars Azmoon (Tehran, Iran). TAC and GSH kits were purchased from ZellBio (GmbH, Ulm, Germany). The essential oil of ZM Boiss was purchased from Barij Essence Pharmaceutical Company (Kashan, Iran). All other chemical materials were of analytical grade and obtained from Sigma-Aldrich (St. Louis, Mo, USA).

Animals

Fifty-one male Sprague-Dawley rats, 200-250g body weight and 6 to 8 weeks of age, were obtained from Comparative and Experimental Medical Center, Shiraz, Iran. The animals were housed in clean cages, maintained in a temperature-controlled environment (25±2°C) with a natural light/dark cycle and given free access to water and a balanced diet.

Animal ethics

All the experiments on animals were approved by the

State Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). The recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the standards in the protection of animals used for experimental purposes were also followed.

Animal modeling

The experimental groups and the doses of ZM Boiss essential oil and CCl_4 were selected based on our previous study (Barghi et al., 2019). After acclimation, the animals were weighed and divided randomly into five groups: Control group (C, n=7): was fed *ad libitum* with normal rat chow and had free access to water; Control oil group (vehicle group, CO, n=7): received 0.2 ml/kg of olive oil orally by intra-gastric gavage, once daily for 11 weeks; Control essential oil group (CE, n=7): received 500µl/kg of ZM Boiss essential oil, diluted with olive oil at a V/V ratio of 60% orally by intra-gastric gavage, once daily for 11 weeks; Fibrosis group (F, n=15): were injected intraperitoneally with CCl_4 at a dose of 1ml/kg body weight diluted 60% (V/V) in olive oil, twice a week for 11 weeks; Fibrosis essential oil group (FE, n=15): received both CCl_4 and essential oil of ZM Boiss (three days before CCl_4 injection) via the manner and doses described for CE and F groups.

Preparation of kidney homogenate

Twenty-four hours after the last administration, under anesthesia, the animals were quickly dissected and their kidney tissues were removed. They were washed immediately with ice-cold saline to remove as much blood as possible. Kidney homogenate was prepared in cold 100mM potassium phosphate buffer (pH=7.4) using a sonication device on ice. Homogenates were centrifuged at 10,000 rpm for 15min at 4°C for removing cell debris. Afterwards, the supernatants were stored at -80°C for later analysis.

Preparation for histopathological study

Regarding the histopathological studies, a portion of each kidney was fixed in 10% formalin buffer, dehydrated and embedded in paraffin wax. The tissues were subjected to the normal routine histological procedures and then stained with hematoxylin and eosin (H&E) to observe kidney injury and Masson's trichrome for detection of possible renal fibrosis.

Evaluation of biochemical and oxidative stress parameters in the kidney homogenates

The activity of AST, ALT, ALP, GGT and LDH enzymes as well as TAC and GSH levels were determined according to the procedures described by the manufacturer's instructions for available kits. Enzymes activities were expressed as the specific activity. TBARS concentration was calculated via the method described (Peeri et al., 2012). In this method, thiobarbituric acid (TBA) reacts with lipid peroxidation products to form a colored product (TBARS) which can be detected using a colorimetric method. To prepare the TBA reagent, 0.375% TBA and 15% trichloroacetic acid were mixed with 37.7% of hydrochloric acid. The samples were mixed with the reagent, placed in a boiling water bath for 15min and, after cooling, centrifuged at 1000rpm for 10min. The absorbance of the supernatant was measured at 535nm and TBARS was calculated using its molar extinction coefficient (1.56×10^5 cm^{-1}/mmol) in equation $A=\varepsilon CL$. The results were expressed as pmol/mg tissue.

Determination of total protein in the tissue homogenates

The protein content of the homogenates was determined by the method of Bradford (Bradford, 1976). Calibration curves were used with crystalline bovine serum albumin.

Statistical analysis

For each parameter considered in the study, the statistical analysis was conducted using one-way ANOVA among groups followed by the post-hoc Tukey test and the data were reported as a mean±SD (using software SPSS 16.0). The *P*-values, <0.05 were regarded as statistically significant.

Results

Biochemical parameters

As shown in Table 1, there is no significant difference among groups in the activity of AST ($P>0.05$). However, the activity of ALT was significantly increased in groups F and FE as compared to the control groups (C, CO and CE, $P<0.05$). Also, the activities of ALP and LDH were significantly increased in group F when compared to the control groups (C, CO and CE). Treatment with ZM Boiss essential oil (group FE) caused a significant reduction in the elevated CCl_4-induced activities of

TABLE 1: The effect of ZM Boiss essential oil on biochemical parameters in kidney tissues of experimental groups.

Groups	AST (mU/mg protein)	ALT (mU/mg protein)	ALP (mU/mg protein)	GGT (U/mg protein)	LDH (U/mg protein)
C	21.82±5.64^{a}	20.63±5.20^{a}	6262.96±1063.59^{a}	4.490.88^{ab}	22.48±5.20^{a}
CO	30.17±6.96^{a}	19.96±7.11^{a}	8053.72±1046.97^{a}	5.04±1.09^{ab}	24.43±5.04^{a}
CE	29.84±7.55^{a}	20.22±6.80^{a}	9187.92±659.33^{a}	5.93±0.41^{a}	20.77±3.53^{a}
F	30.37±3.74^{a}	41.34±12.59^{b}	113322.60±19178.18^{b}	4.56±0.75^{ab}	38.19±6.39^{b}
FE	28.09±5.13^{a}	35.82±9.93^{b}	69614.47±7923.367^{c}	4.25±0.91^{b}	18.85±2.65^{a}

C: control group; CO: control oil group; CE: control essential oil group; F: fibrosis group; FE: Fibrosis essential oil group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transferase; LDH: Lactate dehydrogenase. Data were reported as mean±SD. The different letters in each column indicate a significant difference (P<0.05).

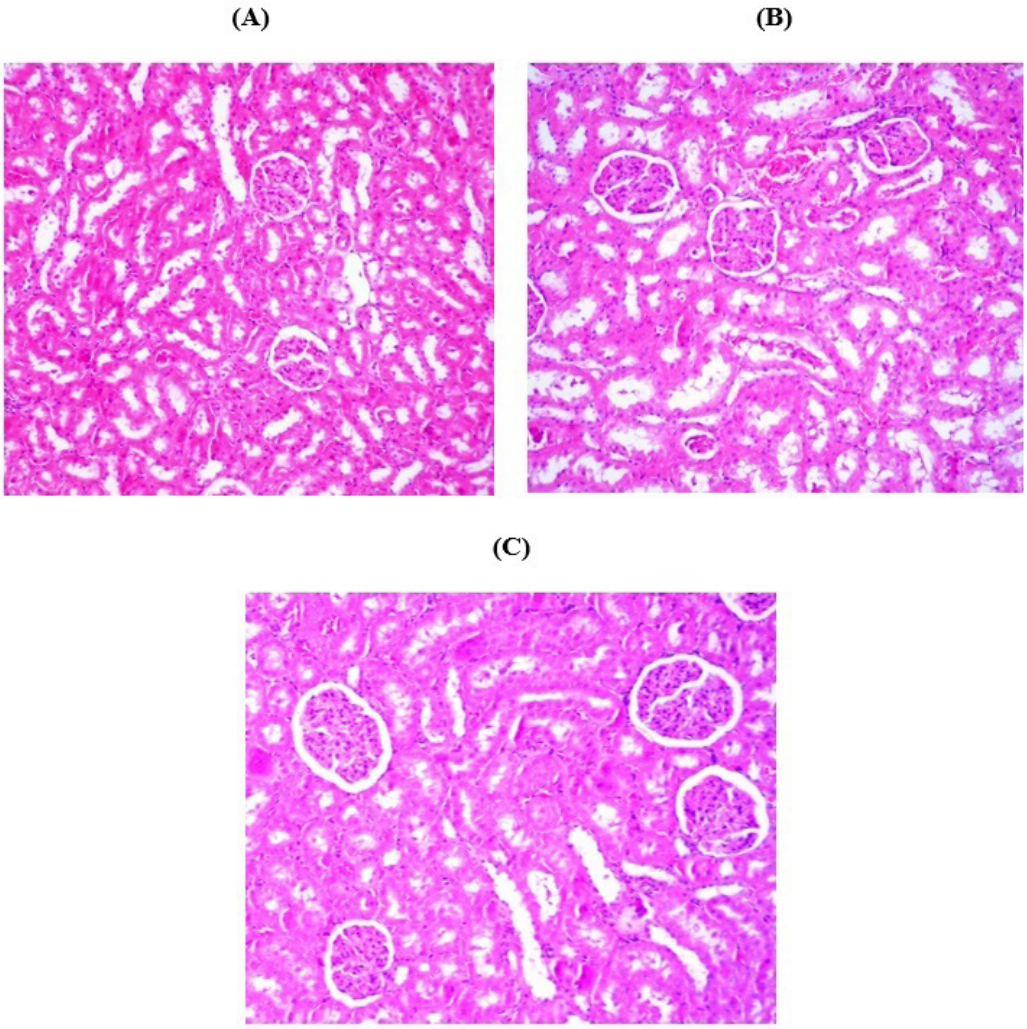

FIGURE 1. Light microscopic analysis of a histopathological section of kidney slices with H&E staining taken from the (A): C group, (B): F group, (C): FE group and (D): F group with Masson's trichrome staining without any significant histopathological alterations. Scale bar=55µm.

ALP and LDH (P<0.05). There was no significant difference in the activity of GGT among groups except for a statistically decreased level of GGT in the FE group as compared to the CE group (P<0.05).

Oxidative stress parameters

Data in Table 2 shows that the content of reduced glutathione in kidney homogenate was significantly increased by ZM Boiss essential oil treatment (group FE)

TABLE 2: The effect of ZM Boiss essential oil on oxidative stress parameters in kidney tissues of experimental groups.

Groups	TAC (µmol/mg tissue)	GSH (mg/mg protein)	TBARS (pmol/mg tissue)
C	1.37±0.77^{a}	17.06±3.39^{a}	32.74±3.16^{a}
CO	3.39±0.87^{ad}	22.42±8.58^{ac}	30.56±3.97^{ac}
CE	4.20±2.01^{bd}	34.11±5.79^{bcd}	23.46±2.98^{bc}
F	6.01±1.09^{b}	27.35±3.93^{ad}	30.28±6.80^{ac}
FE	9.01±1.15^{c}	44.94±10.01^{b}	20.60±3.85^{b}

C: control group; CO: control oil group; CE: control essential oil group; F: fibrosis group; FE: Fibrosis essential oil group. TAC: Total antioxidant capacity; GSH: Reduced glutathione; TBARS: Thiobarbituric acid reactive substances. Data were reported as mean±SD. The different letters in each column indicate a significant difference (P<0.05).

TABLE 3: A review of studies on CCl_4 induced toxicity in tissues.

References	Dose of CCl_4	CCl_4 administration routes	Duration of CCl_4 administration	Animal model	CCl_4-induced toxicity in tissues
Ozturk et al., 2003	1 ml/kg	Subcutaneously	Four consecutive days	Sprague-Dawley rat	Kidney
Khan et al., 2010	3 ml/kg as 30% (V/V) in olive oil	Intraperitoneally	Twice a week for four weeks	Sprague–Dawley rat	Kidney
Ganie et al., 2011	1 ml/kg as a 50% (V/V) solution in olive oil	Intraperitoneally	A single dose	Wistar Albino rat	Kidney and lung
Rahmat et al., 2014	1.5 ml/kg as a 20% V/V in olive oil	Intraperitoneally	A single dose	Wistar Albino rat	Liver and kidney
Sayed et al., 2014	0.5 ml/kg as a 20% V/V in corn oil	Intraperitoneally	A single dose	Swiss Albino mice	Liver and kidney
Safhi, 2018	1.5 ml/kg as 50% (V/V) solution in olive oil/ paraffin oil	Intraperitoneally	Twice a week for 15 days	Swiss Albino mice	Kidney

as compared to the fibrosis group (F, P<0.05). Although there was no significant difference in the concentration of lipid peroxidation marker (TBARS) between F and control groups (C, CO and CE), its level was significantly reduced in the group treated by ZM Boiss essential oil (FE) as compared with group F (P<0.05). Also, the level of TAC was significantly increased in group FE when compared with the fibrosis group (F, P<0.05).

Histopathological changes

As shown in Figure 1, histopathological studies of the control groups demonstrate that the glomerulus and interstitium have the normal architecture. No significant histopathological alterations with H&E and Masson's trichrome staining were observed in photomicrographs of the kidneys after administration of CCl_4 and treatment with ZM Boiss essential oil (groups F and FE, Fig. 1).

Discussion

CCl_4 metabolism occurs in CYP450-dependent pathways. During these processes, two radicals, including trichloromethyl radical (CCl_3•) and trichloromethyl peroxyl radical (CCl_3O_2•) are produced (Safhi, 2018). These radicals can damage tissues including the liver and kidneys by oxidative stress. CYP450 enzymes are abundant in the proximal tubule cells. Therefore, kidney tissues can be susceptible to oxidative damage caused by CCl_4 metabolism. Al-Sayed and Abdel-Daim (2014) used an intraperitoneal injection of CCl_4 (0.5 ml/kg diluted 20% V/V in corn oil) in mice to induce liver and kidney injuries. In the CCl_4-receiving groups, liver and kidney function tests showed that both types of tissues were damaged by CCl_4 due to the induction of oxidative stress by free radicals from CCl_4 origin. Studies by other researchers, for instance, that of Safhi (2018), on kidney

toxicity and CCl_4 induced oxidative damage have shown that injecting this substance into mice (with dose 1.5 ml/kg diluted 50% in olive oil injected intraperitoneally twice a week for 15 weeks) can cause oxidative stress in kidney tissues. Damage to kidney tissue by induction of oxidative stress from CCl_4 has also been shown in other studies. Ganie et al. found that intraperitoneal injection of CCl_4 (a single dose of 1 ml/kg diluted 50% in olive oil) can damage the kidney and lung tissues in rats by producing free radicals (Ganie et al., 2011). In addition to the studies mentioned above, Table 3 shows a number of other relevant studies in which renal tissue damage following the administration of CCl_4 was confirmed by histopathological findings, biochemical tests to evaluate renal function, and evaluation of antioxidant and oxidant status. The data in this table show the differences in doses of injection, dilution ratios of CCl_4 in oil, durations of injection and species of laboratory animals, as compared to our study. The results of the present study showed that chronic administration of CCl_4 caused changes in the activity of some enzymes in kidney tissues. GGT enzyme has a crucial role in the metabolism of glutathione (a potent antioxidant). It can reflect oxidative stress and glutathione consumption (Sepulveda, 2019). Therefore, in the present study, the lack of change in the activity of this enzyme along with no significant changes in TBARS level (as a marker of lipid peroxidation) in groups treated with CCl_4, as compared to the control groups, may indicate the steady state of oxidative balance without any damage to kidney tissues, as the histopathological results did not show destructive changes in kidney tissues in group F compared to control groups. However, the findings of our previous study showed that the injection of the same dose of CCl_4 intraperitoneally can induce oxidative stress and fibrosis in liver tissues (Barghi et al., 2019). It is probable that, in addition to difference of dosage and duration of administration, another factor that may be responsible for the difference in the incidence of toxicity in the liver and kidney tissues, is the difference in CYP450 protein expression in these tissues since, as was previously mentioned, CCl_4 metabolism by CYP450 initiates tissue damage. Research suggests that the expression of CYP2E1, a member of the CYP450 family and an important enzyme involved in the detoxification of xenobiotics, is controlled by several factors such as the species of animal models and the types of inducing agents (Song and Cederbaum, 1996; Goasduff et al., 1996). The expression of CYP2E1 can be different due to intoxication in liver, kidney and lung tissues (Zerilli et al., 1995). Therefore, further studies should also examine the type of CYP450 and its expression in liver and kidney tissues following CCl_4 poisoning. In the present study, the effect of CCl_4 on AST, ALT, ALP and LDH activity in group F did not follow a definite pattern, but may reveal changes in cell metabolism due to CCl_4 injection, which may alter the expression and synthesis of some enzymes. Some researchers have shown that liver damage induced by the *administration* of CCl_4 causes a change in Krebs cycle and gluconeogenic fluxes, which subsequently results in changes in the activity of some enzymes involved in these pathways both in liver and kidney tissues (Carvalho et al., 2002). For example, it has been shown that following renal ischemia, the concentration of pyruvate is reduced following the conversion to lactate by LDH (Zager et al., 2014). Zager et al. revealed changes in the activity of renal cortical LDH in the damage caused by ischemia with renal toxicants being considered as a diagnostic factor. Thus, to provide a definitive conclusion, future research should evaluate the concentration of lactate and pyruvate as well as the activity of enzymes such as pyruvate dehydrogenase, pyruvate kinase, phosphoenolpyruvate carboxy kinase in the kidney and serum of rats that receive CCl_4 (Zager et al., 2014).

In the present study, changes in the TAC and GSH levels in CE and FE groups indicate the efficacy and the antioxidant properties of ZM Boiss essential oil. In addition to being a popular spice and seasoning, ZM is also used as an antimicrobial, anti-inflammatory and antioxidant, to name a few, in traditional medicine (Barghi et al., 2019). The certificate of analysis for ZM Boiss essential oil prepared by the central laboratory of the Barij Essence Pharmaceutical Company, Kashan, Iran, shows that thymol and carvacrol are two main constituents of this essential oil, which make up 31.10% and 27.49% of the extract, respectively. Thymol and carvacrol, as phenolic monoterpens, are responsible for the antimicrobial, anti-inflammatory and especially antioxidant properties of ZM Boiss essential oil (Kavoosi and Rabiei, 2015; Sajed et al., 2013; Sharififar et al., 2011). Similar results about the antioxidant properties of ZM have been reported by other researchers (Raeisi et al., 2018; Shomali et al., 2016). Also, the antioxidant properties of ZM Boiss essential oil have been investigated

in food preservation by other researchers (Behnam and Aliakbarlou, 2014).

Conclusion

Although administration of CCl_4 at a dose of 1ml/kg body weight that is diluted 60% (V/V) in olive oil, intraperitoneally, twice a week for 11 weeks affected on the activity of some biochemical enzymes in kidney tissues, the results of oxidative stress and histopathological evaluation showed that there was no oxidative stress or injury in kidney tissues. However, prophylactic administration of ZM Boiss essential oil had antioxidant properties in modulating the measured parameters.

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Conflict of interest

The authors declare that they have no competing financial interests.

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